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WITNESS my hand this
Tenth day of June 2003

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PROVISIONAL SPECIFICATION

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Invention Title: **Cell Fusion**

The invention is described in the following statement:

CELL FUSION

Background of the Invention

The present invention relates to a method and apparatus for fusing first and second cells, and in particular, for producing hybrid cells by electrofusion.

Description of the Prior Art

The reference to any prior art in this specification is not, and should not be taken as, an acknowledgement or any form of suggestion that the prior art forms part of the common general knowledge in Australia.

Previously it has be known to fuse cells together using a variety of techniques such as chemical fusion employing polyethylene glycol, biological methods such as viruses or viral proteins or electrofusion of cells in suspension. Methods of fusion carried out by chemical or biological means often suffer from problems associated with contamination, low efficiency and cytotoxity.

There are a number of advantages to using electrofusion for producing hybrid cells. The fusion conditions can be better controlled and optimised depending on the type of cell to be fused than chemical or biological fusion allows. This allows electrofusion to lead to an increase in cell fusion efficiency.

The basis for electrofusion is to expose pairs of cells, in close membrane contact, to an electric field that induces a sufficient voltage across their cellular membrane to cause mechanical breakdown of the cell membrane and the formation of pores at the point of cell-to-cell contact. Ideally the pores should be of sufficient size to allow transfer of cellular material between the two cells, particularly allowing the two nuclei to come together and subsequently fuse. This formation of pores should also be reversible such that any pores formed at points other than that of cell-to-cell contact seal quickly.

A further process known as dielectropherisis (DEP) in which a non-uniform alternating

electric field is applied to the cells in order to ensure good cellular contact prior to fusion. Typically DEP is employed to form 'pearl chains' of large numbers of cells between the electrodes in the fusion container.

Whilst electrofusion has a number of advantages over chemical and biological processes, all of these methods fuse cells simultaneously in large numbers. The pairing of the cells, necessary to form hybrid cells; is therefore completely random. Accordingly, the current methods are limited in that they require a chemically sensitive immortal cell such that unfused and self-fused cells can be eliminated from the final culture.

Furthermore, these 'bulk' methods also do not lend themselves to hybrid creation from rare cells. Typically fusion methods require millions of cells in order to overcome the problems associated with ensuring cellular contact between the desired cells. In some cases the number of target cells for fusion might only number in 10's-100's. Further, in the recovery phase, whereby cells from the fusion process are plated out and the chemical selection process to remove unfused cells takes place, there is no guarantee of clonal purity in the final product. This plating of cells is also extremely time consuming.

It would therefore be desirable to provide a method whereby lesser numbers of cells could be selected, electrically fused and recovered to grow.

An example of a system suitable for performing electrofusion on a small number of cells is described in WO93/05166. This describes apparatus that utilises an electrode coated with ligands. In use, the ligands are used to attract target cells bearing complimentary ligands. Once the ligands are bound, the cells are therefore effectively bound to the electrode. Accordingly, at this point the target cells can be brought into contact with partner cells allowing the cells to be fused.

However a number of drawbacks exist with these techniques. Firstly, the target cell is held in contact with the electrode during the electrofusion process. As a result the cell is usually subject to an intense electric field which tends to damage the cell. Secondly, the

technique can only be performed with a number of target cells attached to the electrodes, and a number of partner cells. Accordingly, this means that any cells successfully fused may be separated out from cells that do not fuse, which can be a complex procedure. A further disadvantage of this technique is that cells can bind to the electrode non-specifically leading to false fusion events taking place.

A second example of a system for performing cell fusion on individual cells is described in WO01/09297. In this example, cells are manipulated using a combination of optical trapping, and pushing the cells with micro-electrodes. Once the cells are correctly positioned relative to each other, an electric field is applied to the cells to cause the cells to fuse.

However, a number of significant drawbacks exist with the apparatus. Firstly, the presence of the laser and associated optics required to manipulate the cells results in the apparatus being expensive, time consuming to configure and complicated to use. Secondly, the electrodes must be significantly smaller than the cells in size, to allow manipulation of the cells by pushing. As a result, the electrodes are again expensive, difficult to construct and extremely fragile, thereby further increasing the cost and complexity of the apparatus.

In addition to this, touching the cells with the electrodes can lead to additional problems, such as burning of the cells. Even in the event that a signal is not being applied to the electrodes when the cell is pushed, the electrodes can retain a residual field from when they are last used. In this case, contact of the cell with the electrode can cause the field to be discharged, thereby damaging the cell.

Finally, the use of the laser trapping and electrodes to manipulate cells is difficult to achieve manually as described in WO01/09297. This not only means that training is required to perform cell fusion using the apparatus, but also means the cell fusion process itself can be time consuming.

Summary of the Present Invention

In a first broad form the present invention provides a method of fusing first and second cells, the method including:

a) Selecting the first and second cells;

b) Positioning the first and second cells between two electrodes in a fluid filled fusing container, the first and second cells being held in suspension separated from each electrode; and,

c) Applying a current having a predetermined waveform to the electrodes to cause the cells to fuse.

The method of selecting the first and second cells typically includes using a pipette to extract:

a) The first cell from a group of first cells held in a first container; and,

b) The second cell from a group of second cells held in a second container.

The method of positioning the first and second cells between the two electrodes usually includes:

a) Using the pipette to position the first cell in the fusing container;

b) Using the pipette to position the second cell in the fusing container, adjacent the first cell;

c) Positioning the electrodes such that the first and second cells are located substantially between the electrodes.

The pipette is typically coupled to:

a) A drive system adapted to move the pipette with respect to the first, second and fusing containers; and,

b) An actuator adapted to actuate the pipette to thereby expel or draw in fluid through a port.

In this case, the method usually includes using a controller coupled to the drive system and the actuator to move and actuate the pipette.

The method of selecting a cell preferably includes causing the controller to:

a) Move the pipette such that the port is adjacent a cell having predetermined characteristics, the cell being held in fluid suspension in the respective container;

b) Actuate the pipette to draw in fluid through the port, thereby drawing in the cell and the surrounding fluid.

The method of using the pipette to position the second cell adjacent the first cell generally includes causing the controller to:

a) Move the pipette such that the port is adjacent the first cell in the fusing container;

b) Cause the pipette to expel fluid through the port, thereby expelling the second into the fluid in the fusing container;

c) Move the pipette such that the port is as close as possible to both the first and second cells;

d) Cause the pipette to draw in fluid through the port, thereby drawing in the first and second cells and the surrounding fluid;

e) Cause the pipette to expelling the first and second cells into the fluid in the fusing container; and,

f) Repeat steps (c) to (e) until the first and second cells are within a predetermined distance.

The electrodes may be coupled to an electrode drive system adapted to move the electrodes with respect to the fusing containers, in which case the method typically includes using a controller coupled to the electrode drive system to position the electrodes in the fusing chamber.

The electrodes may be coupled to a waveform generator, in which case the method of applying the alternating current includes causing the waveform generator to apply a predetermined waveform to the electrodes.

If the first and second cells having a respective cell type, the method preferably includes using a controller coupled to the field generator to select the waveform in accordance with the cell types of the first and second cells.

The fist and second cells may be the same type of cell, the first and second group of cells being the same group.

In a second broad form the present invention provides apparatus for fusing first and second cells, the apparatus including:

a) A fluid filled fusing container;
b) At least two electrodes adapted to be positioned in the fusing container in use;
c) A selector for:
   i) Selecting a first cell from a group of first cells held in a respective container; and,
   ii) Selecting a second cell from a group of second cells held in a respective container;
   iii) Positioning the first and second cells in the fusing container, the first and second cells being held in suspension; and,
d) A field generator coupled to the electrodes, the field generator being adapted to cause a field having a predetermined waveform to be generated between the electrodes, thereby causing the cells to fuse.

The selector is preferably a pipette.

The apparatus generally further includes:

a) A drive system adapted to move the pipette with respect to the first, second and fusing containers; and,
b) An actuator adapted to cause the pipette to expel or draw in fluid through a port.

The apparatus generally further includes an electrode drive system adapted to move the electrodes with respect to the fusing containers.

The apparatus also typically includes a controller adapted to control the fusing of the cells by controlling operation of at least one of:

a) The pipette;

b) The electrodes; and,

c) The field generator.

The controller typically includes a processor coupled to at least one of:

a) The drive system and the actuator, the processor being adapted to move and actuate the pipette;

b) The electrode drive system, the processor being adapted to move the electrodes; and,

c) The field generator, the processor being adapted to cause the field generator to generate the field having the predetermined waveform.

The controller may include a detector adapted to detect the position of cells within the containers, in which case the processor can be responsive to the detector to move at least one of the electrodes and the pipette in response to the position of detected cells.

Alternatively, or additionally, the processing system may include an input for receiving input commands from a user.

The processor can be coupled to a store for storing waveform data representing a number of different predetermined waveforms, the processor being adapted to select one of the number of predetermined waveforms in response to the input commands received from the user.

The processor can also being adapted to move at least one of the electrodes and the pipette in response to the input commands received from the user.

Typically the controller is adapted to cause the cells to fuse by causing the apparatus to perform the method of the first broad form of the invention.

In a third broad form the present invention provides, a controller for controlling apparatus for fusing first and second cells, the apparatus including:

a) A fluid filled fusing container;

b) At least two electrodes;

c) A selector;

d) A field generator coupled to the electrodes;

Wherein, in use, the controller is adapted to cause the cells to fuse by:

i) Causing the selector to:

(1) Select a first cell from a group of first cells held in a respective container; and,

(2) Select a second cell from a group of second cells held in a respective container; and,

(3) Position the first and second cells in the fusing container, the first and second cells being held in suspension;

ii) Positioning the electrodes in the fusing container; and,

iii) Causing the field generator apply a field having a predetermined waveform to the electrodes, thereby causing the cells to fuse.

In this case, the controller typically includes a processor coupled to at least one of:

a) A drive system adapted to move the pipette with respect to the first, second and fusing containers;

b) An actuator adapted to cause the pipette to expel or draw in fluid through a port;

c) An electrode drive system adapted to move the electrodes with respect to the fusing containers; and,

d) The field generator.

The controller is typically adapted to operate for use with apparatus of the second broad form of the invention.

In this case, the controller is preferably adapted to cause the apparatus to perform the method of the first broad form of the invention.

In a fourth broad form the present invention provides a computer program product for controlling apparatus for fusing first and second cells, the computer program product including computer executable code which when executed by a suitable processing system causes the processing system to operate as the controller of the third broad form of the present invention.

Brief Description of the Drawings

An example of the present invention will now be described with reference to the accompanying drawings, in which: -

Figure 1 is a block diagram of an example of apparatus for implementing the present invention;

Figure 2 is a schematic diagram of the apparatus of Figure 1;

Figure 3 is a schematic diagram of the pipette of Figure 1;

Figure 4 is a flow chart of an overview of the process implemented by the apparatus of Figure 1;

Figures 5A to 5C are a flow chart of an overview of the process implemented by the apparatus of Figure 1;

Figures 6A and 6B are schematic diagrams of cells being drawn into and expelled from the pipette of Figure 3;

Figures 6C and 6D are schematic diagrams of the arrangement of the electrodes and cells in the fusion well during operation of the apparatus of Figure 1; and,

Figures 7A to 7G are examples of pulse sequences that may be used in the apparatus of Figure 1.

Detailed Description of the Preferred Embodiments

An example for apparatus suitable for implementing the present invention will now be

described with reference to Figures 1, 2 and 3.

As shown in Figure 1, the apparatus includes a processing system 10 coupled to an imaging system 11, a control system 12 and a signal generator 13. The control system 12 is coupled to a pipette system 14 and an electrode system 15, as shown.

The processing system 10 includes a processor 20, a memory 21, an input/output (I/O) device 22, an image interface 23, a control interface 24, and a signal interface 25, coupled together via a bus 26. The processing system may therefore be any one of a number of systems, such as a suitably programmed computer, specialised hardware, or the like. In any event, the I/O device typically includes a display, such as a computer monitor or the like, a keyboard, and one or more other input devices such as a mouse, joystick, trackball or the like.

The imaging system 11 includes a camera 30 such a CCD camera or the like which is coupled to a microscope 31. The imaging system 11 is connected to the processing system via the image interface 23.

The pipette system 14 includes a pipette shown generally at 33 that is coupled to the control system 12 via a drive system 32. In use, the control system 12 is coupled to the processor via the control interface 24, thereby allowing the drive system 32 to be used to control motion and operation of the pipette, as will be described in more detail below.

Similarly, the electrode system 15 is formed from two electrodes 35 coupled to the control system 12 via a drive system 34. Again, the control system 12 allows the drive system 34 to control the position of the electrodes, as will be described in more detail below.

In use, the system allows a user to select and move individual cells using the pipette system 14. When appropriate cells are placed next to each other, this allows an electric field to be applied to the cells using the electrodes 35 thereby causing the cells to fuse.

In order to achieve this, the apparatus is arranged as shown schematically in Figure 2 such that the pipette 33 and the electrodes 35 may be moved relative to a well array shown generally at 40. This allows cells to be moved between the wells 40, 41, 42, 43, 44, 45, 46, 47, 48, as shown.

Movement of the pipette and the electrodes 35 is achieved by operation of the corresponding drive system 32, 34. Accordingly, it will be appreciated that the processing system 10 may be used to control positioning of the pipette 33 and the electrodes 35 allowing the pipette 33 and the electrodes 35 to be inserted into and positioned within a respective one of the wells 41, ..., 48.

Furthermore, the microscope 31 is arranged to image selected wells 41, ..., 48 such that the representation of the contents of a selected well can be displayed to the user using the I/O device 22.

In general, the processing system 10 is adapted to control the pipette 33 and the electrodes 35 in accordance with input commands received from the user via the I/O device 22. In order to achieve this, the processing system 10 must be able to perform a number of functions simultaneously, such as:

- Presenting an image of the well array 40 to the user on the I/O device 22;
- Responding to commands input via the I/O device 22 to move and, if required, actuate the pipette system 14;
- Responding to command inputs via the I/O device 22 to move the electrodes 35; and,
- Responding to commands input via the I/O device 22 to apply an electrical signal to the electrodes 35.

This is achieved by having the processor 20 execute appropriate application software which is stored in the memory 21.

The pipette is shown in more detail in Figure 3. As shown, the pipette 33 is formed from a

housing 50 defining a chamber that is divided into two portions 51A, 51B by a piezo-electric element 52, as shown. The chamber 51B is coupled by a port 53 to a flexible tube 54. The flexible tube 54 includes a male coupling 55 that is adapted to cooperate with a female coupling 56 positioned on a shaped glass nozzle 57 having an aperture 58, as shown.

In use, the chamber 51B, the port 52, the flexible tube 53 and the glass nozzle 54 are filled with fluid, with the chamber 51A being filled with air and sealed. Applying a current to the piezo-electric element 52, via leads 59, causes the element to move, with the direction of movement depending on the polarity of the applied current.

Thus, in use, with the aperture 59 positioned in fluid in one of the wells 41, ..., 48, causing the piezo-electric element 52 to move in the direction of the arrow 60 will increase the volume of the chamber 51B, thereby causing fluid to be drawn through the aperture 59. Similarly, causing the piezo-electric element 52 to move in the direction of arrow 61 will decrease the volume of the chamber 51B, thereby causing fluid to be expelled through the aperture 59.

Accordingly, the pipette can be activated to draw in or expel fluid through the aperture 58 depending on the polarity of the current applied to the leads 56. Accordingly, in use, the leads 56 are coupled to either the drive system 32, or a separate activation system, to allow a suitable current to activate the pipette as required.

The manner in which the apparatus is used to fuse cells will now be described.

Overview

An overview of the method of fusing cells in accordance with the present invention will now be described with reference to Figure 4.

In particular, at step 100, the user selects the cells to be fused. At step 110, the cells are placed in a fusion well.

At step 120 a predetermined electric field is applied to the selected cells to cause the cells to fuse.

Cells that are placed in an electric field will distort the field in their immediate vicinity. The field distortion is dependent on the geometry and electrical properties of the particle and that of the surrounding particles. Living cells have interior (cytoplasm) that is highly conductive, due to the accumulation of ions such as potassium (K+) ions, and a relatively high dielectric constant. The membrane surrounding has a very low conductivity and a lower dielectric constant.

Accordingly, the degree of the distortion of the field both inside and outside of the cell is a very strong function of the frequency of the applied electric field. As a result when placed in a non-uniform electric field cells will experience a force whose magnitude and direction will vary in a complicated manner with the frequency of the applied field. This effect can be exploited to selectively manipulate living cells using radio-frequency alternating electric fields created via suitable electrodes. The movement of particles in AC electric fields is referred to as 'dielectropherisis' (DEP) and is independent of any net charge on the particle.

The application of the radio-frequency electric fields exerts a positive DEP force on the two cells, urging the cells into close contact with each other. A stronger electric field is then used in order to induce electrical breakdown of each cell's membranes at their point of contact. This controlled electro-poration triggers a process of cell fusion that is somewhat akin to reverse-mitosis. This in turn creates a fused hybrid cell that has a genetic makeup that is a combination of the two original cells that were fused.

The fused cell is then generally placed in a recovery well at step 130 before being checked after a predetermined time period to confirm the cell has fused at step 140.

The fused cells can then be collected at 150 and used as required.

Detailed Description

A detailed example of the method of using the apparatus of the present invention will now be described with reference to Figures 5A, 5B, 5C and 5D.

In this example, the well array 40 includes a target well 41, a partner well 42, a washing well 43, a fusion well 44, a recovery well 45 and a hybrid well 46 the purpose of which will be described in more detail below.

At step 200 the target and partner cells are placed in respective target and partner wells. This procedure will generally involve suitable preparation of the cells, which may be achieved in a number of manners. Thus, for example, this may require that the cells are recovered from sample plates and washed in appropriate enzyme solutions.

The well array would then be sterilised before appropriate fluids are inserted into the wells to be used. The target and partner cells are then placed in the target and partner wells, 41, 42 respectively, with the cells being held in suspension in respective enzyme solutions.

At step 210, the user selects a target cell from the target well 41 using the pipette 33. In order to achieve this, the user will arrange the well array 40 such that the target well 41 is imaged by the imaging system. Accordingly, the target well 41 is placed under the microscope 31 so that the camera 30 may generate an image signal and transfer this to the image interface 23. The image signal will then generally undergo some pre-processing in the image interface 23 before being transferred to the processor 20 for any subsequent further processing.

Thus, for example, the image interface 23 may be formed from an image capture card, which is used to capture images from incoming image signals. The captured image is then formatted by the processor 20 before being presented to the user using the I/O device 22.

The user adjusts the relative position of the microscope 31 and the well array until a

suitable target cell is shown. The user then uses the processing system 10 to control the position of the pipette 33. In particular, this is usually achieved by having a joystick I/O device 22, with the processor 20 responding to signals from the joystick to generate commands which are transferred via the control interface 21 to the control system 12. The control system will typically be formed from a motion control addressing amplifier, which is coupled to a drive system 32, such as suitable stepper or DC servo motors.

By use of appropriate sensitivity control, this allows the position of the pipette to be controlled to high degree of accuracy. By arranging the microscope such that the pipette is shown in the image presented on the display, this allows the user to position the pipette 33 with the pipette aperture 58 adjacent the selected cell.

At this point, the user activates the pipette 33 to draw fluid in through the aperture 58. The cell and the surrounding fluid will be drawn into the pipette, allowing the target cell to be removed from the target well 41.

Sometimes, it is difficult to separate individual cells within the wells. This can be overcome by repeatedly operating the pipette to cause the pipette to repeatedly draw in and expel fluid via the pipette aperture 58. Agitation of the fluid medium and repeated movement of the cells through the pipette aperture 58 will usually allow a cell to be separated from surrounding cells.

An example of this is shown in Figure 6A, which shows the hydrodynamic stream-lines 70 as fluid is expelled from the pipette aperture 58. As shown, the hydrodynamic stream-lines, which represent lines of constant force, spread out away from the pipette aperture 58. Similarly, as the cells, shown at 71, 72, are entrained in the fluid flow, this will tend to cause the cells 71, 72 to separate as they are expelled away from the pipette aperture 58.

In any event, once the user has selected the target cell at step 210, the user washes the target cell in a fusion medium in the washing well 43. In order to do this, the pipette containing the respective cell is positioned in the washing well 43, using the imaging and

control system 11, 12 to move the pipette 33 as described above. Once the pipette 33 is positioned inside the washing well 43, the pipette is repeatedly activated to cause fluid to be drawn in through and expelled through the pipette aperture 58. In this way, the cell is repeatedly placed in the fusion medium in a washing well 43 and then removed. This action causes the cell to be washed.

Furthermore, when the user transfers the target and cell to the fusion well 44 at step 230, this is achieved by positioning the pipette 33 in the washing well 43 and drawing the target and cell into the pipette 33 through the pipette aperture 58. Accordingly, at this point the target cell is surrounded in fusion medium as opposed to in the medium contained in the target well 41.

The user then uses the pipette 33 to place the target cell into the fusion well 44 at step 230. Steps 210 to 230 are repeated for the partner cell, with the partner cell being placed in the fusion well 44 next to the target cell at 230.

As an alternative to performing steps 210 to 230 separately for each cell, the target and partner cells may be selected from the respective wells and then washed together in the washing cell 43 being transferred simultaneously to the fusion well 44.

As will be described in more detail below, it is preferable for the cells 71, 72 to be positioned adjacent to each other. In order to achieve this, it is preferable to first place the target or partner cell 71 in the fusion well 44 and then place the other partner or target cell 72 adjacent thereto.

In general as adding the second cell 72 will cause fluid to be transferred into the fusion well 44, this also causes movement of the first cell 71. It is then generally necessary to repeatedly activate the pipette 33 until the both cells can be drawn in to the pipette simultaneously. As shown in Figure 6B, when the cells 71, 72 are drawn in to the pipette aperture simultaneously, the hydrodynamic lines of force 70 converge as the fluid enters the aperture 58. Accordingly, this draws the cells 71, 72 together. The cells can then be

expelled from the pipette 33 with the cells being sufficiently close for the fusion process to be performed.

In any event, once the user has positioned the target and partner cells in the fusion well at 230 the user then arranges to place the electrodes 35 in the fusion cell 44 at step 240. Again, in order to achieve this, the imaging system 11 is positioned such that the I/O device 22 presents the user with an image of the fusion cell 44.

The user can then alter the position of the electrodes 35 by providing appropriate commands via the I/O device 22. Again, this is usually achieved by having a respective joystick or the like provide control signals to the processor 20. The processor then transfers appropriate command signals via the control interface 24 to the control system 12. The control system then activates the drive system 34, thereby casing the electrodes 35 to move as directed by the user.

An example of the relative positioning of the electrodes 35, the cells 71, 72 and the pipette 33 at this stage is shown in Figures 6C and 6D, which show a perspective and end on view of the fusion well 44 prior to fusion being performed. Thus, as shown, the cells 71, 72 are positioned close to each other substantially between the electrodes 35. At this stage the cells need not be in contact as they will in any event be urged together by the applied electrical field as will be described in more detail below.

As shown in Figure 5B, the next step is for the user to determine the sequence of electric fields that are to be applied to the cells at step 260 before using the processing system 10 and the signal generator 13 to generate the determined pulse sequence at step 270.

The manner in which the user determines the electric field will vary depending on the particular implementation of the invention. A first example by which this may be achieved is shown in steps 280, 290. In this case, the processing system 10 applies a predetermined electric field to the partner and target cells. The response of the cells in the electric field is then used to determine the electrical parameters employed for the DEP electric field (in

order to bring the cells together). The response can also be used to determine the fusion pulse sequence (including the frequency and amplitude) required to fuse any particular pair of cells. In particular, the processing system 10 will apply a field having a predetermined frequency. The frequency can then be fine adjusted until an optimum frequency is determined at which the force that attracts the cells to cells move toward each other is optimal for the required conditions. This response of the cells to the DEP electric field will occur due to the generation of electric dipoles within the cells, as described above.

The response of the cells to the electric field can be monitored either automatically by having the processor 20 execute appropriate image recognition software, or manually by the user. The processor would then select a pulse sequence from a number of pulse sequences stored in the memory 32. The pre-programmed pulse sequences would be stored in a look up table (LUT), or the like, in accordance with the field applied to obtain the desired response. It will be appreciated that this information may need to be determined initially. Accordingly, each time a new lineage of target and partner cell combination is fused, the pulse sequence used to achieve this successfully will be stored in the LUT and the memory 21, together with information regarding the complete set of fusion parameters at which the desired response was observed. The processor 20 can then use the indication of the response to select a pulse sequence from the LUT.

Alternatively, the pre-programmed pulse sequences could be stored in the LUT in accordance with each particular type of target and partner cell combination. Again, this information will need to be determined initially. However, by storing the pulse sequence each time a new target and partner cell combination is fused, this allows the processor 20 to select a pulse sequence at step 310 in accordance with cell types provided by the user at step 300.

In any event, the electric pulse sequences applied to the cells to cause the cells to fuse by DEP at step 320.

At step 330, whilst the cells are still in the fusion well 44 the user examines the both

morphology and the electrical behaviour of the cells to determine if they have fused to create a fusate cell. If the morphology and behaviour appear favourable to fusion then the fusate is transferred using the pipette 33 to the recovery well 45 at step 360. The initial stages of cell fusion only take a few minutes, typically under ten for most type of cells and accordingly, the user can simply view the cells on the I/O device 22 and determine from this whether the fusion process has been successful. If it is determined that the cells have not fused at step 340, the user simply discards the unfused cells with the pipette 33 at step 350, and returns to select new cells at step 210.

Once placed in the recovery well 45 the fusate cell is left for approximately 45 minutes before again being checked at step 370. During this time, the cell is held in suspension in a suitable culture medium to encourage cell growth. If it is determined that the fusate cell has not completely fused at step 380 then the user discards the unfused cells using the pipette 33 at step 390, and selects new cells at step 210.

Otherwise, the user transfers the fusate cell to a respective hybrid well 46 using the pipette 33 at step 400. The fusate cell is incubated in the hybrid well at step 410, with the cell being monitored after and during the incubation process at step 420, to determine if the fusion has been successful.

Pulse Sequences

As described briefly above, different pulse sequences may be used to control the fusion of the two cells. The generation of different pulse sequences is achieved by having the processor 20 control the signal generator 13 in accordance with pre-determined pulse sequences stored in the memory 21. The pulse sequences are generally stored in data arrays and associated parameters in an LUT, as outlined above or calculated using suitable equations and data arrays at the point of fusion. The processor 20 extracts the necessary parameters and the like stored in the memory 21 and transfers this information to the signal interface 25.

In this example, the signal interface 25 is in the form of an arbitrary waveform generator or the like, which uses the determined parameters to define a desired pulse sequence. The waveform generator therefore generates a signal representative of the pulse sequence and transfers this to a high frequency signal amplifier, allowing the desired pulse sequence to be transferred to the electrodes 35 as required.

It will be appreciated that other forms of pulse sequence generation can also be used.

In any event, an example of different electrical pulse sequences that may be used for fusing cells will now be described. In each of these examples, the functions are defined in the temporal domain, t

The basic pulse sequence profiles may be defined in terms of the equations:

$$y_1(t) = A\sin(\omega t) \qquad < t_1$$
$$y_2(t) = C(t) \qquad t_1 < t < t_2$$
$$y_3(t) = B\sin(\omega t) \qquad > t_2$$

where: A is a constant

B is a constant (and may be equal to A)

$C(t)$ is the function describing the pulse.

$C(t)$ is typically based on one of the following functions, although it will be appreciated that this is not essential:

$$C_1(t) = \pm K$$
$$C_2(t) = Q\exp(-\alpha t)$$
$$C_3(t) = Q\exp(\alpha t)$$
$$C_4(t) = Q\sin(\xi t)$$

where: K Q, α and ξ are constants.

Basic pulse sequences can be combined and overlaid to create complex sequences, some examples of which are listed below and are shown in Figures 8A to 8G.

Figure 7A shows a first example of a Basic DC Fusion Pulse Sequence consisting of 2 unipolar square pulses, separated by sinusoidal waves. The equations used to govern the generation of these pulse sequences are as follows:

$$\begin{aligned} y_1(t) &= A\sin(\omega t) && < t_1 \\ y_2(t) &= +K && t_1 < t < t_2 \\ y_3(t) &= B\sin(\omega t) && t_2 < t < t_3 \\ y_4(t) &= +K && t_3 < t < t_4 \\ y_5(t) &= A\sin(\omega t) && > t_4 \end{aligned}$$

Figure 7B shows a second example of a Basic DC Fusion Pulse Sequence consisting of a bipolar square pulse, separated by sinusoidal waves. The equations used to govern the generation of these pulse sequences are as follows:

$$\begin{aligned} y_1(t) &= A\sin(\omega t) && < t_1 \\ y_2(t) &= +K && t_1 < t < t_2 \\ y_3(t) &= -K && t_2 < t < t_3 \\ y_4(t) &= A\sin(\omega t) && > t_3 \end{aligned}$$

Figure 7C shows a third example of a Basic AC Fusion pulse consisting of a sinusoidal (of differing frequency) of increased amplitude and differing frequency separated by sinusoidal waves. The equations used to govern the generation of these pulse sequences are as follows:

$$\begin{aligned} y_1(t) &= A\sin(\omega t) && < t_1 \\ y_2(t) &= Q\sin(\xi t) && t_1 < t < t_2 \\ y_3(t) &= B\sin(\omega t) && t_2 < t < t_3 \\ y_4(t) &= Q\sin(\xi t) && t_3 < t < t_4 \\ y_5(t) &= A\sin(\omega t) && > t_4 \end{aligned}$$

Figure 7D shows a fourth example of a Basic DC and exponential pulse separated by sinusoidal waves. The equations used to govern the generation of these pulse sequences are as follows:

$$y_1(t) = A\sin(\omega t) \qquad < t_1$$
$$y_2(t) = +K \qquad t_1 < t < t_2$$
$$y_3(t) = K + Q\exp(-\alpha t) \qquad t_2 < t < t_3$$
$$y_4(t) = A\sin(\omega t) \qquad > t_3$$

Figure 7E shows a fifth example of a Basic DC and exponential pulse separated by sinusoidal waves. The equations used to govern the generation of these pulse sequences are as follows:

$$y_1(t) = A\sin(\omega t) \qquad < t_1$$
$$y_2(t) = +K \qquad t_1 < t < t_2$$
$$y_3(t) = K - Q\exp(\alpha t) \qquad t_2 < t < t_3$$
$$y_4(t) = A\sin(\omega t) \qquad > t_3$$

Figure 7F shows a sixth example of a Basic DC pulse sequence convoluted with a linear curve. The equations used to govern the generation of these pulse sequences are as follows:

$$y_1(t) = A\sin(\omega t) \qquad < t_1$$
$$y_2(t) = +K \otimes (-\beta t) \qquad t_1 < t < t_2$$
$$y_3(t) = B\sin(\omega t) \qquad t_2 < t < t_3$$
$$y_4(t) = +K \otimes (-\beta t) \qquad t_3 < t < t_4$$
$$y_5(t) = A\sin(\omega t) \qquad > t_4$$

Note. An extra DC pulse is shown in Figure 7F for clarity.

Figure 7G shows a seventh example of a Basic DC pulse convoluted with an exponential decay curve. The equations used to govern the generation of these pulse sequences are as follows:

$$y_1(t) = A\sin(\omega t) \qquad < t_1$$
$$y_2(t) = +K \otimes \left(Q\exp(-\alpha t)\right) \qquad t_1 < t < t_2$$
$$y_3(t) = B\sin(\omega t) \qquad t_2 < t < t_3$$
$$y_4(t) = +K \otimes \left(Q\exp(-\alpha t)\right) \qquad t_3 < t < t_4$$
$$y_5(t) = A\sin(\omega t) \qquad > t_4$$

Note. An extra DC pulse is shown in Figure 7G for clarity.

Specific Example

An outline of the production of a human-human hybridoma using the apparatus of Figure 1 will now be described. In general the explanation will focus on the following staged of the process.

- Preparation of the cells for fusion
- Setup of the apparatus for fusion
- Manipulation of the cells in readiness for fusion
- Electrofusion of the selected pair of cells to obtain hybrid fusates.

Preparation of the cells for fusion.

Peripheral Blood Mononuclear Cells (PBMC) were prepared according to the following protocol: Buffy Coats are obtained from healthy donors (sero-negative for HIV, Hep-B, Hep-C, HTLV-I and Syphilis) from the Australian Red Cross Blood Bank, Sydney, NSW. PBMC are isolated by density centrifugation on Ficoll-Paque™ Plus (Amersham Pharmacia, 17-1440-03). The B cells are then isolated for fusion. Untouched B cells are isolated from PBMC with MACS B Cell Isolation Kit (Miltenyi BioTec, 469-01) by magnetic depletion of T cells, NK cells, myeloid cells, basophils, platelets and early erythroid cells. A human myleloma cell line, designated F4 was used as the immortal partner cell.

Set-up of the apparatus for fusion

A 20ml syringe (#1) was loaded with RPMI media (#2) warmed in an incubator (30 mins at 37 C). Using the syringe a large droplet of the warmed RPMI solution was deposited

into the centre of a Petrie dish (#3). This dish was then placed on the inverted microscope (Nikon TE2000) such that it was situated beneath the pipette. The pipette having first been sterilized with repeated washings of 70% alcohol/water solution. The pipette was then lowered so that the tip was immersed in the droplet of RPMI. One end of a length of silicon tubing(#4) (with suitable connectors(#5)) was attached to a second syringe and the other end to the pipette. RPMI was then gently drawn into the pipette and through the tubing using the syringe. Care was taken to ensure that no air bubbles formed anywhere along the tubing or in the pipette. Using the RPMI filled syringe, fluid was injected into the nozzle of the piezo electric actuator until it was completely filled and a positive meniscus formed over the nozzle. The second syringe was then gently uncoupled from the silicon tubing. Using the first syringe filled with RPMI the uncoupled end of the silicon tubing was topped with fluid until a positive meniscus over the mouth of the connector. The tubing was then coupled to the piezo electric nozzle 54.

Each pipette nozzle 54 is drawn from capillary tubing (120μm inner diameter) from (#7)

The electrodes 35 were then aligned using a graticule until they were spaced ~400 – 500 μm apart.

The previously prepared partner cells were then transferred to a single well in a 96 well plate (#6) and the lymphocytes were deposited in a separate well in the same plate.

The pipette was then inserted into the well containing the partner cells and a suitable partner cell selected. This (single) cell was then transferred to a fresh well containing RPMI + 10% fetal calf serum; FCS. The pipette was then inserted into a well containing the previously sorted B lymphocytes specific to the target antigen. A suitable B lymphocyte for fusion was then selected. Returning to the previous well the lymphocyte cell was expelled from the pipette beside the partner cell. Both cells were then visually inspected for their suitability for fusion.

Manipulation of the cells prior to fusion

The pipette was then used to transfer both cells into a well containing a sorbitol solution of appropriate pH and osmolarity. The cells were then 'washed' in the fusion media by gently inhaling and expelling them through the pipette aperture 58 in order to allow them to acclimatise to the changed environment. Once the cells had adjusted to the change in osmolarity the pipette was then used to hydro-dynamically arrange the cells so that they were within 5-10μm of each other. The pipette was then removed from the well.

The electrodes 35 were inserted into the well and arranged so that the previously arranged cells lay centred and co-linearly between them. Each electrode is constructed from a nickel alloy wire of 180μm diameter manufactured by the Californian Fine Wire Company, California, USA. The configuration of the electrodes, their shape and their proximity to the cells are specifically designed so that a suitable electric field pattern can be generated in order to induce DEP between the cells.

The electrodes were connected through an amplifier to the arbitrary waveform generator and a series of voltages conforming to different waveforms, previously defined by the user, were applied. The first waveform applied to the electrodes was sinusoidal and had a frequency of 500 kilohertz and an amplitude, post amplifier, of approximately 6V peak to peak. Through phenomena known as dielectropherisis, whereby neutral particles become polarised in the presence of an alternating, non-uniform, electric field, the cells experienced a force of attraction that caused them to coalesce.

The amplitude of the field was then increased to 15V peak to peak for a period of 5 seconds ensure that good membrane contact was made between the cells. In this increased field there was a slight drift of the cells towards the upper electrodes, and to counter this the stage of the microscope was adjusted relative to the electrodes to correct and retain the cells position between the electrodes.

Electrofusion of the selected pair of cells to obtain hybrid fusates.

Once the cells were suitably arranged a field pattern conforming to the DC pulse sequence was applied. In this instance the DC pulse sequence consisted of two pulse trains, each train consisting of 2 DC pulses, of amplitude 90V, (resulting in an electric field of approximately 180kV) each being of 80μs duration. The pulses were separated by a duration of 100μs, and each train was separated by 500 milliseconds, during which in the intervening time a DEP was applied in order to keep the cells in good contact. Post fusion pulse sequence, an increased DEP field was applied in order to maintain good contact between the cells whilst the cells fused.

Recovery of the cells to growth medium

The electrodes 35 were the retracted from the fusion well, and the pipette 33 was inserted and manipulated so that the newly created fused cells were in the vicinity of the pipette aperture 58. The cells were then inhaled into the pipette and the pipette retracted from the well. In this fashion the cells were transferred to a fresh well containing hybridoma growth media (RPMI + 10% FCS). The newly fused cells were the only cells that were present in this media.

Automation

The above description focuses on manual use of the apparatus, in which positioning of the cells, electrodes and pipette are controlled in accordance with commands input by the user.

However, alternatively the processing system 10 can be adapted to control the apparatus automatically. In order to achieve this, the processor 20 executes image recognition applications software stored in the memory 21. This allows the processing system to use images received from the imaging system 11 to determine the position of cells within the wells 41, ... 48, as well as to determine the position of the electrodes 33 and the pipette 33.

From this, it will be appreciated that the processor 20 and be programmed to perform the procedure outlined above automatically. Accordingly, the processing system will be adapted to automatically select target and partner cells in accordance with the appearance

of the cell in the image. The cells will then be placed in the fusion well 44 to allow the fusion to be performed. Again, during this process the processor 20 will control the position of the cells and the electrodes.

The processor then determines the pulse sequence to be applied to the cells, and applies the pulse sequence via the electrodes 35. Once this is completed the processor 20 can monitor the cells to determine if the fusion process is successful.

Accordingly, the system described above allows individual cells to be easily fused. As the cells are manipulated using the pipette as shown in Figure 3, this makes the cell manipulation far easier than in the prior art. This therefore helps increase the speed and ease with which fusion of individual cells can be performed. Furthermore, the electrodes need never touch the cells, thereby helping reduce or prevent cell damage prior to or during the fusion process.

In addition to this, the apparatus as a whole is generally less complicated, thereby helping reduce the cost, as well as easing use of the apparatus to perform cell fusion. As a result, fusion using the system described above can generally be achieved more rapidly and cheaper than in the prior art.

Persons skilled in the art will appreciate that numerous variations and modifications will become apparent. All such variations and modifications which become apparent to persons skilled in the art, should be considered to fall within the spirit and scope that the invention broadly appearing before described.

Accordingly, while the above description has focused on cell fusion, it will be appreciated that the techniques may generally be applied to any cells, vectors, particles, molecules, liposomes, and other such vesicles.

Dated this Nineteenth Day of June, 2002

APOLLO LIFE SCIENCES PTY LIMITED

By their Patent Attorneys

DAVIES COLLISON CAVE

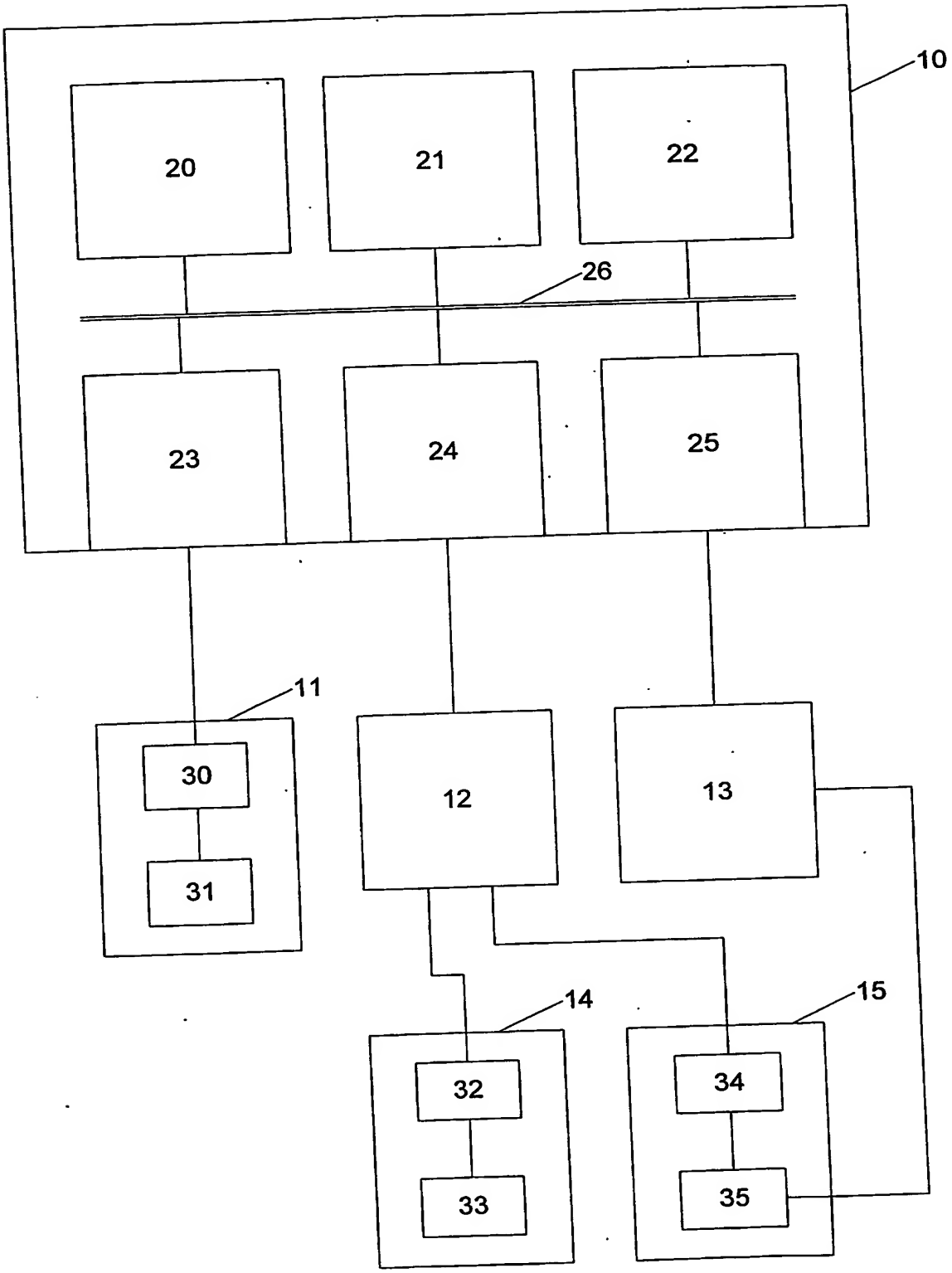

Fig. 1

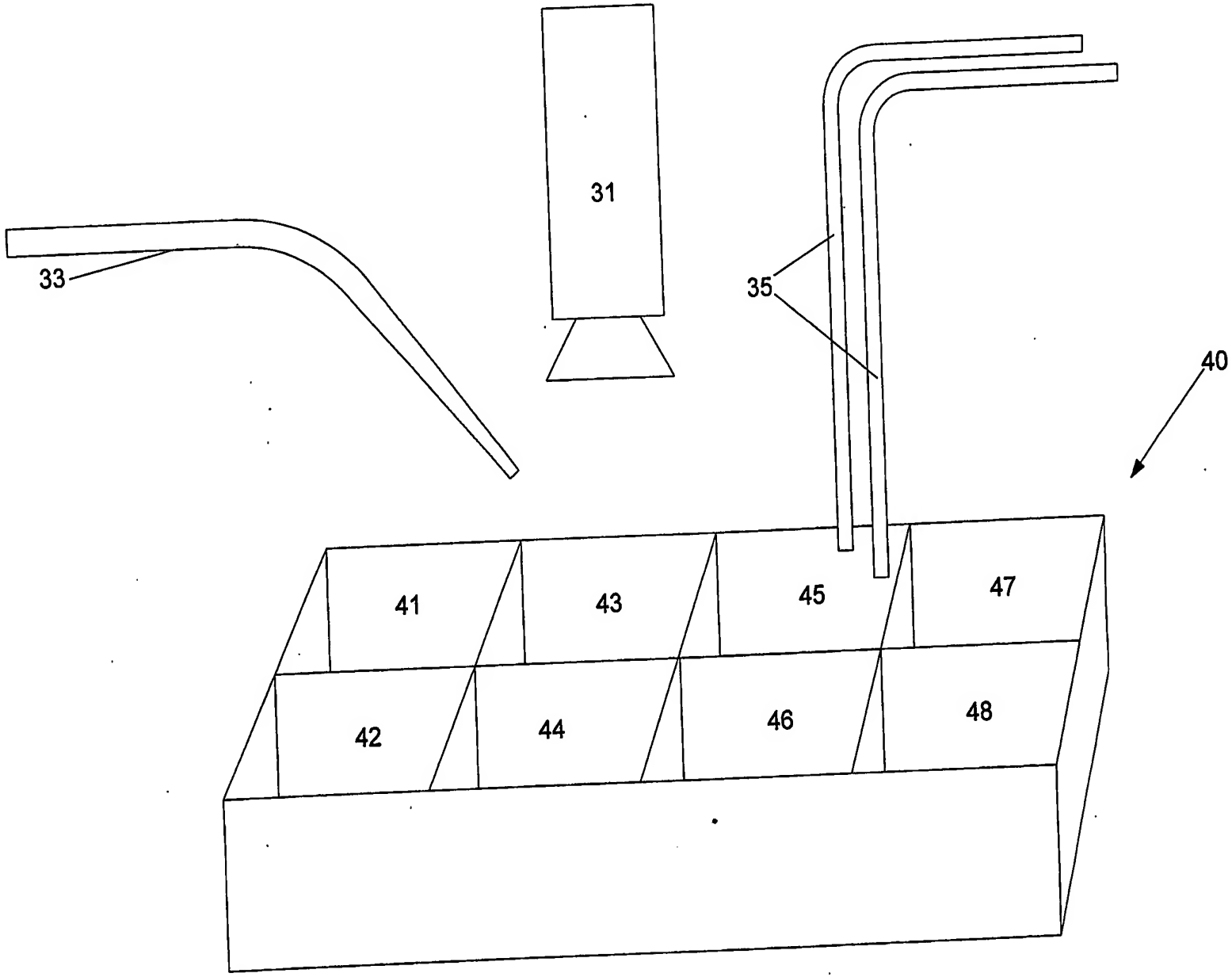

Fig. 2

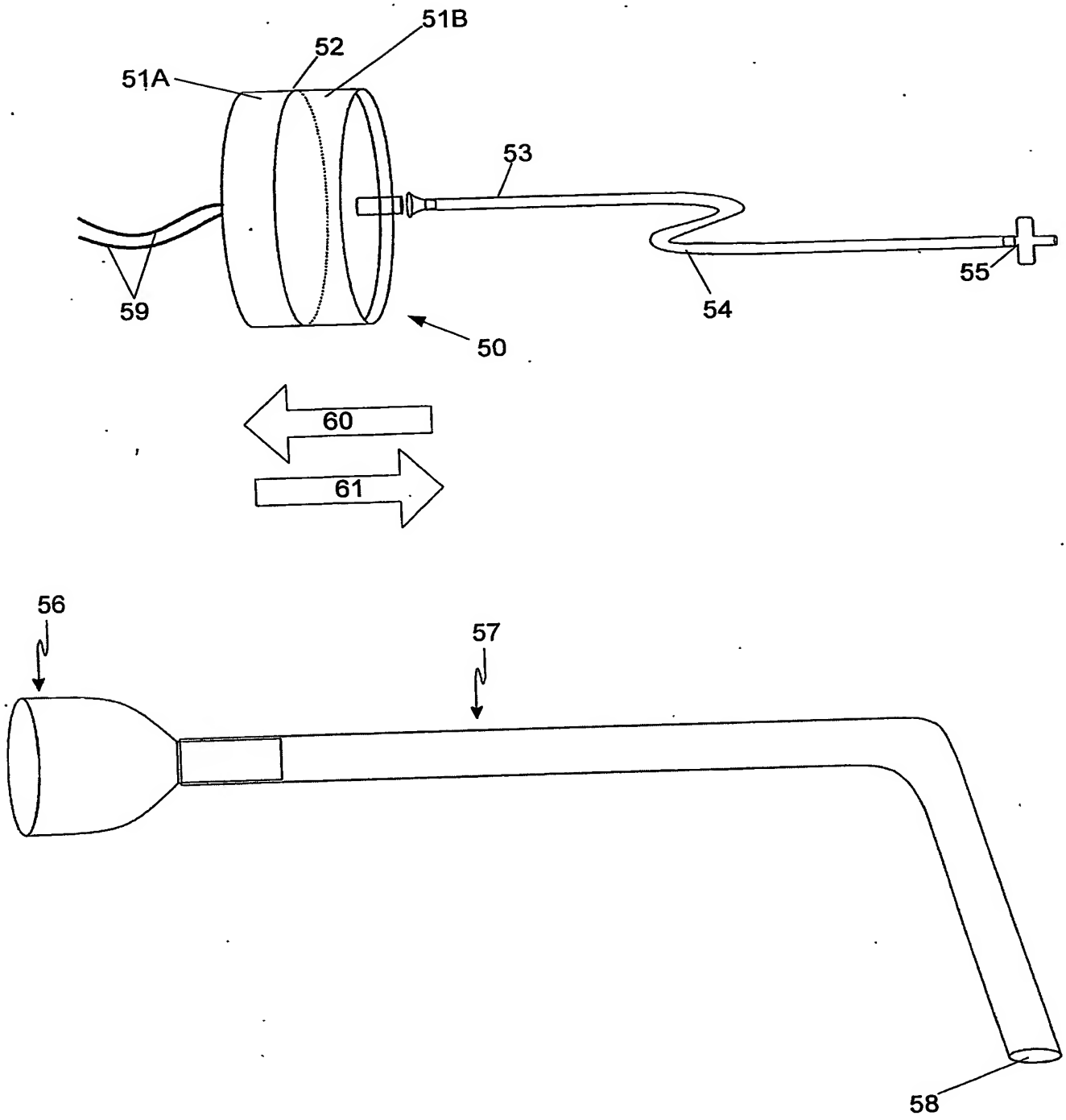

Fig. 3

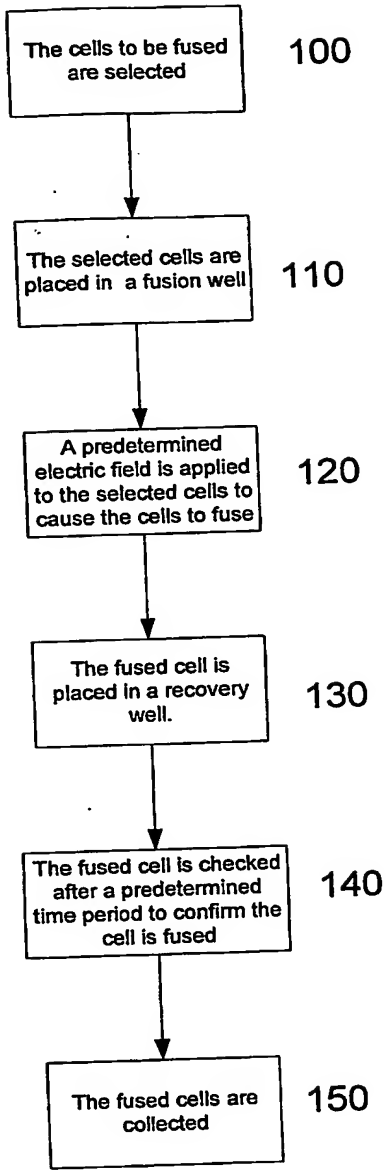
The cells to be fused are selected
100
The selected cells are placed in a fusion well
110
A predetermined electric field is applied to the selected cells to cause the cells to fuse
120
The fused cell is placed in a recovery well.
130
The fused cell is checked after a predetermined time period to confirm the cell is fused
140
The fused cells are collected
150

Fig. 4

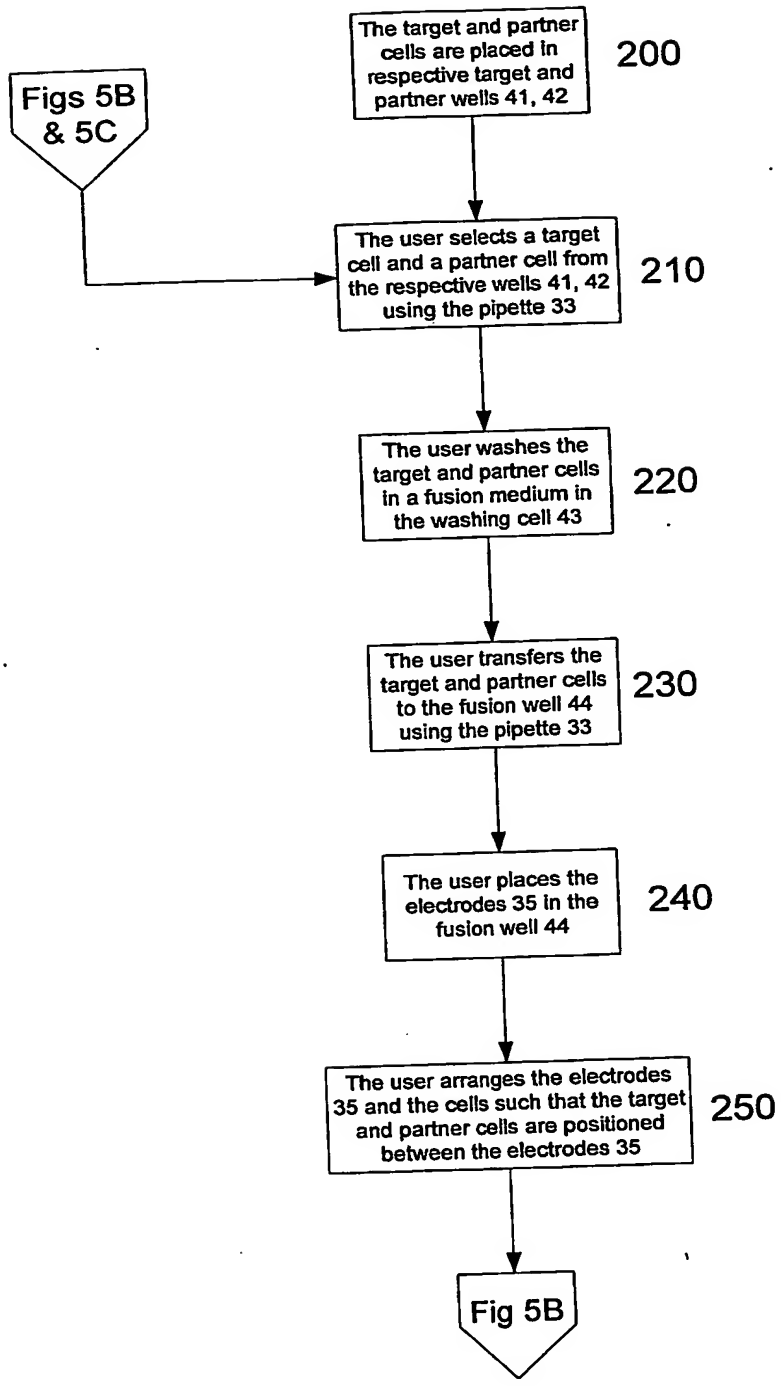
Figs 5B & 5C
The target and partner cells are placed in respective target and partner wells 41, 42
200
The user selects a target cell and a partner cell from the respective wells 41, 42 using the pipette 33
210
The user washes the target and partner cells in a fusion medium in the washing cell 43
220
The user transfers the target and partner cells to the fusion well 44 using the pipette 33
230
The user places the electrodes 35 in the fusion well 44
240
The user arranges the electrodes 35 and the cells such that the target and partner cells are positioned between the electrodes 35
250
Fig 5B

Fig. 5A

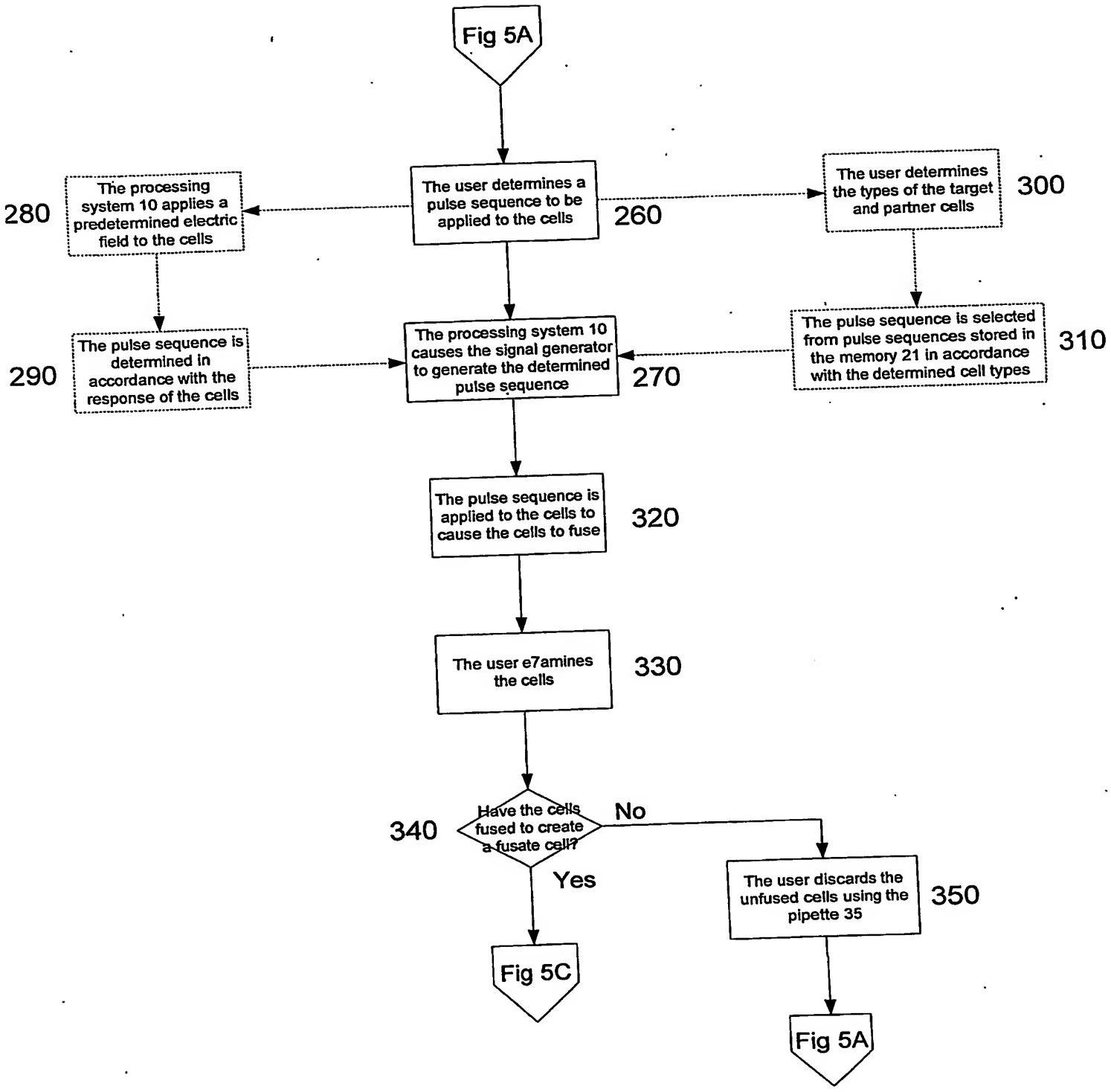
Fig 5A
The user determines a pulse sequence to be applied to the cells
260
The processing system 10 applies a predetermined electric field to the cells
280
The user determines the types of the target and partner cells
300
The pulse sequence is determined in accordance with the response of the cells
290
The processing system 10 causes the signal generator to generate the determined pulse sequence
270
The pulse sequence is selected from pulse sequences stored in the memory 21 in accordance with the determined cell types
310
The pulse sequence is applied to the cells to cause the cells to fuse
320
The user e7amines the cells
330
Have the cells fused to create a fusate cell?
340
No
Yes
The user discards the unfused cells using the pipette 35
350
Fig 5C
Fig 5A

Fig. 5B

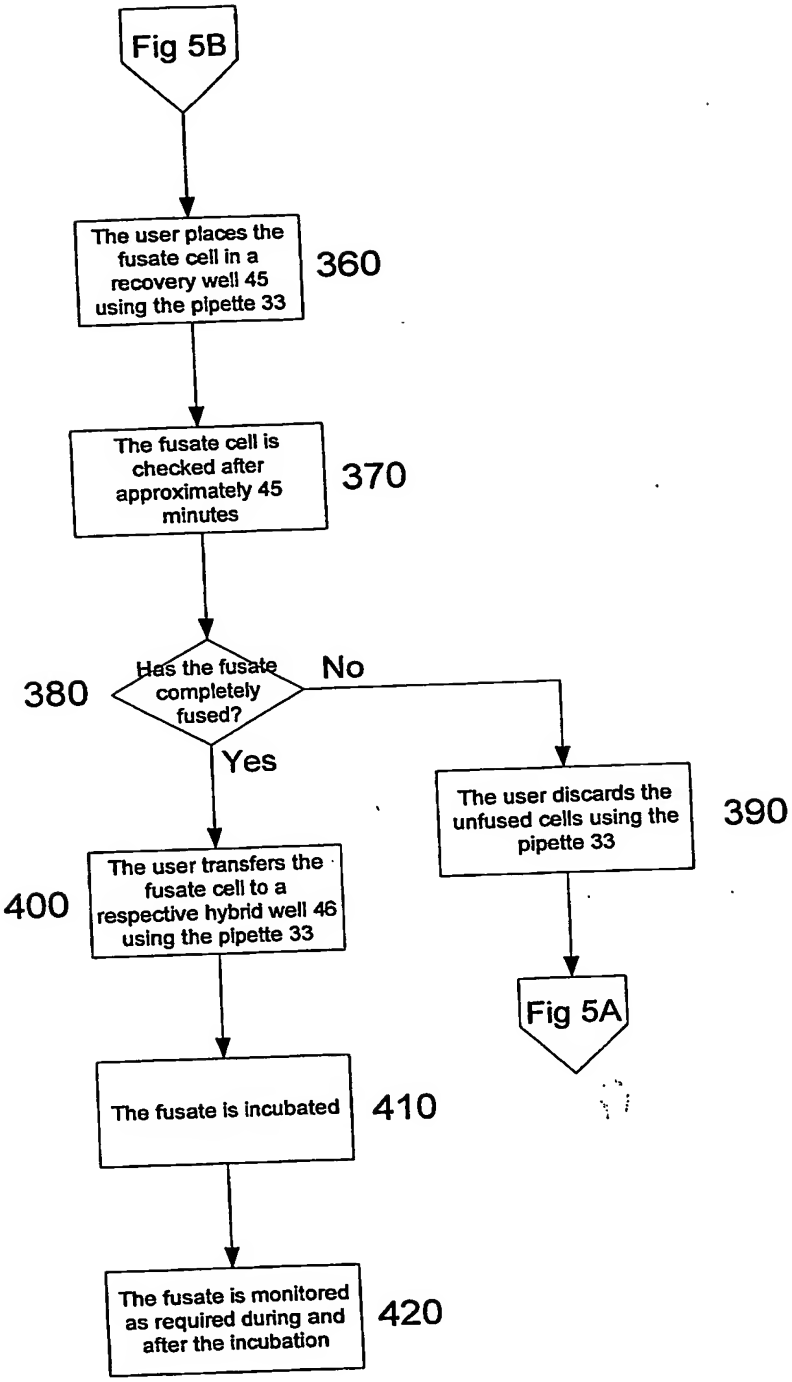
Fig 5B
The user places the fusate cell in a recovery well 45 using the pipette 33
360
The fusate cell is checked after approximately 45 minutes
370
380
Has the fusate completely fused?
No
Yes
The user discards the unfused cells using the pipette 33
390
Fig 5A
400
The user transfers the fusate cell to a respective hybrid well 46 using the pipette 33
The fusate is incubated
410
The fusate is monitored as required during and after the incubation
420

Fig. 5C

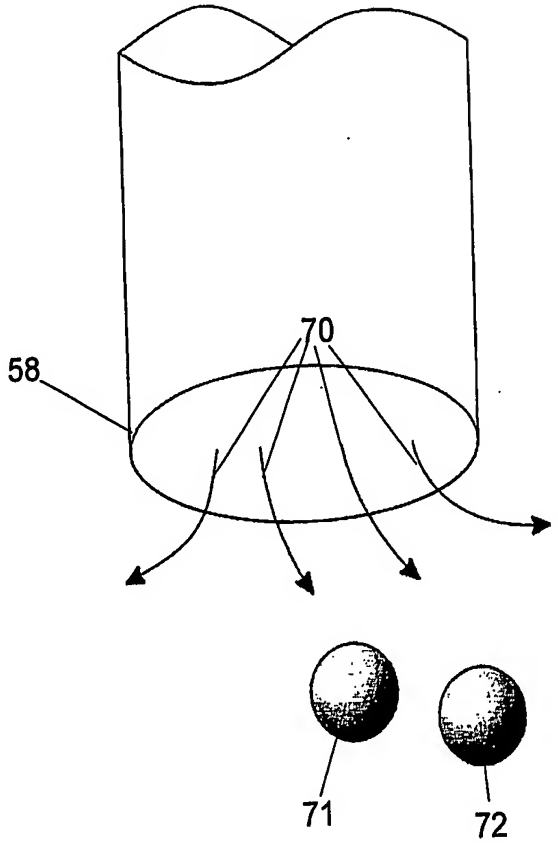

Fig. 6A

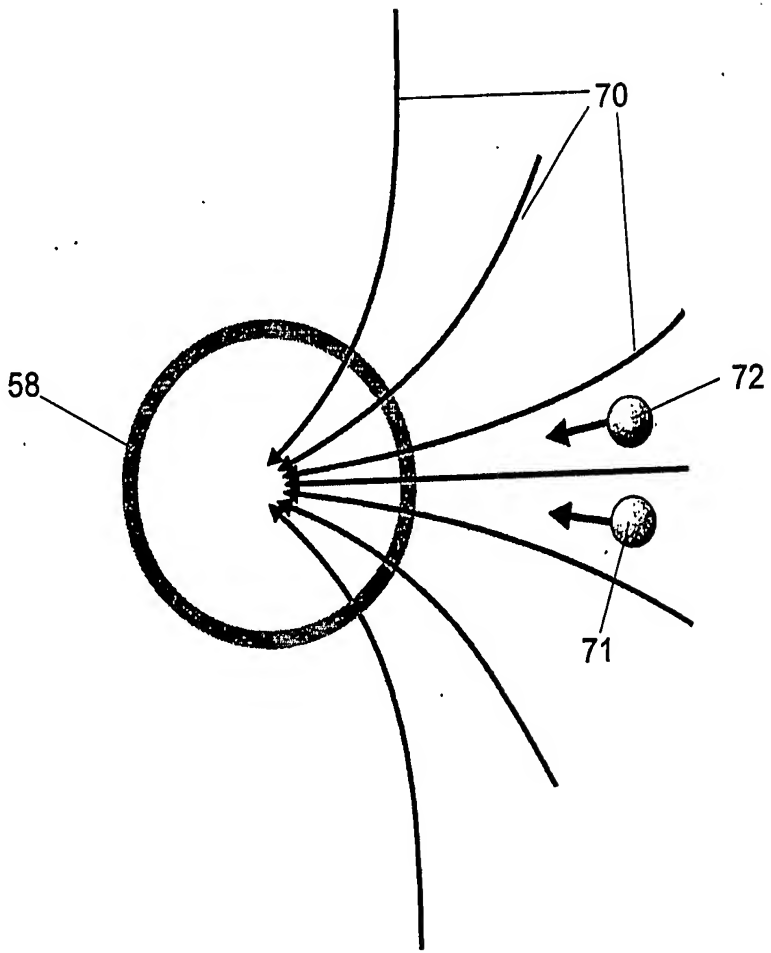

Fig. 6B

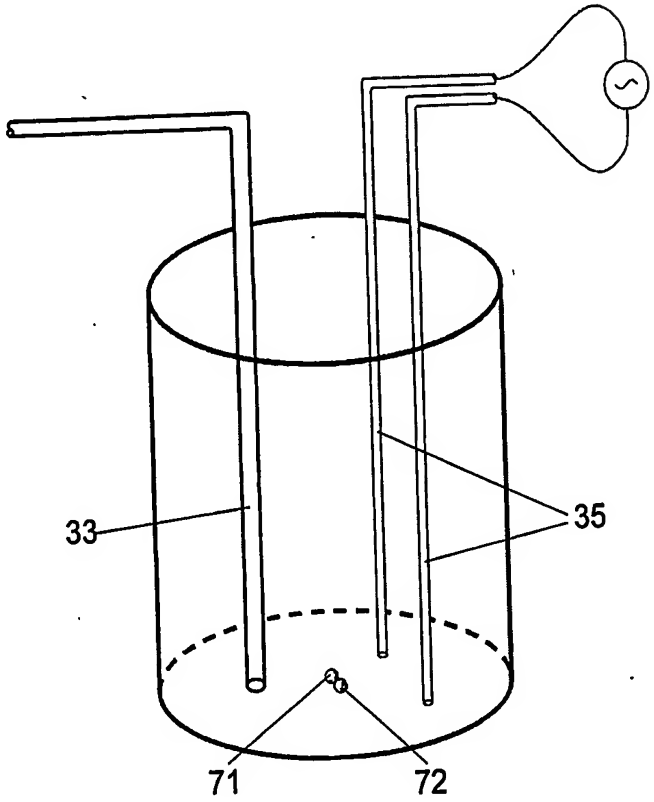

Fig. 6C

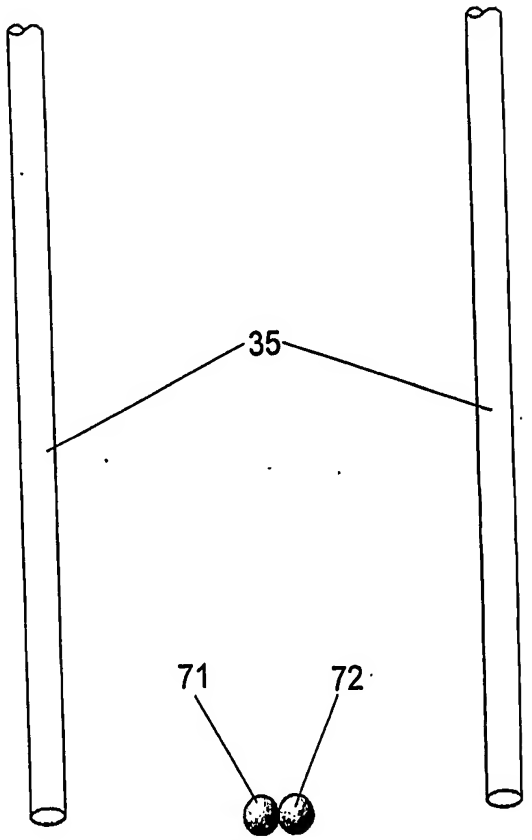

Fig. 6D

Fig. 7A

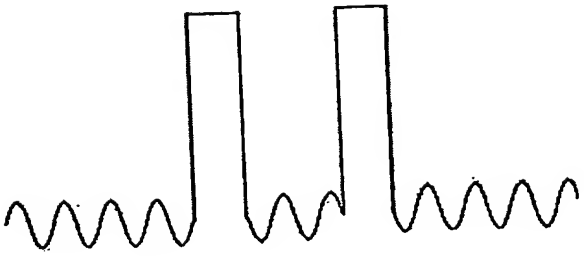

Fig. 7B

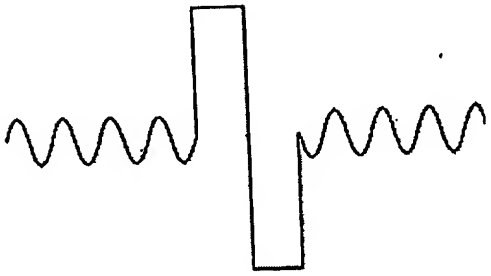

Fig. 7C

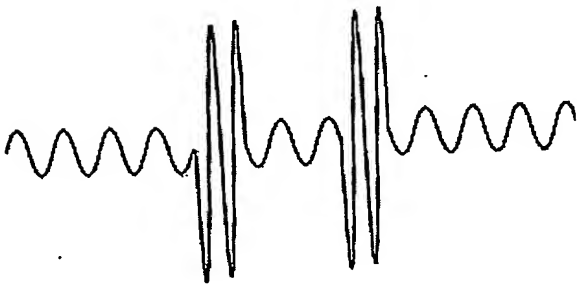

Fig. 7D